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First Identification of 4-*S*-Glutathionyl-4-methylpentan-2-one, a Potential Precursor of 4-Mercapto-4-methylpentan-2-one, in Sauvignon Blanc Juice

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The identification of 4-*S*-glutathionyl-4-methylpentan-2-one (glut-4-MMP) by high-performance liquid chromatography−tandem mass spectrometry (HPLC−MS/MS) experiments in a Sauvignon Blanc juice extract is described. Synthesis of an authentic reference compound enabled confirmation of the presence of glut-4-MMP in a Sauvignon Blanc juice for the first time. The juice extract was obtained by batch-wise percolation of 6 L of juice through a sintered glass funnel packed with C18 sorbent, followed by further purification using low-pressure chromatography on C18. Analysis of the juice extract revealed a chromatographic peak with the same retention time and mass spectrum as the synthetic reference compound, and spiking experiments verified the findings. The presence of glut-4-MMP in grape juice may be related to the biosynthesis of the relevant *S*-cysteinyl conjugate and, subsequently, to the formation of aroma-active 4-mercapto-4-methylpentan-2-one (4-MMP). This compound has a very low reported sensory threshold (3 ng/L) in wine and is partially responsible for the aromas that are important to the quality and style of some wine varieties.

KEYWORDS: Thiol precursors; *S*-glutathionyl conjugates; wine aroma; HPLC−MS; isolation; identification; synthesis

INTRODUCTION

Volatile thiols are widely recognized as important contributors to the aroma of some wine varieties (*1*−*4*). In particular, three thiols, 4-mercapto-4-methylpentan-2-one (4-MMP), 4-mercapto-4-methylpentan-2-ol (4-MMPOH), and 3-mercaptohexan-1-ol (3-MH) (**Figure 1**), responsible for box tree, citrus zest, and grapefruit scents, respectively (*1*, *2*), are not present in grape juice as free thiols but are released from nonvolatile precursors during alcoholic fermentation (*5*, *6*). Tominaga and co-workers (*5*) first identified *S*-cysteinyl conjugates of these thiols in a Sauvignon Blanc juice. More recently, Peyrot des Gachons et al. (*7*) tentatively identified the presence of the glutathione conjugate of 3-MH (**Figure 1**) in Sauvignon Blanc juice. The identification was based on low- and high-resolution mass spectrometry (MS), where the correct $[M + H]^+$ ion (*m*/*z* 408) and molecular formula for 3-*S*-glutathionylhexan-1-ol (glut-3MH) were observed. The disappearance of the MS signal at *m*/*z* 408 upon treatment of the sample with γ-glutamyltranspeptidase and an increase in the intensity of an MS fragment attributed to 3-*S*-cysteinylhexan-1-ol (cys-3MH) also indicated the presence of the glutathione conjugate of 3-MH.

The tripeptide glutathione is very important in the interactions of biological systems with their environment. In grape berries, the reduced form is the most abundant free thiol-containing (−SH) compound at harvest. However, the glutathione present in the fruit rapidly disappears after crushing, because of the beginning of redox and enzymatic processes (*8*). The formation of glutathione conjugates in plants and animals can be ascribed to processes related to detoxification of xenobiotic and endog-

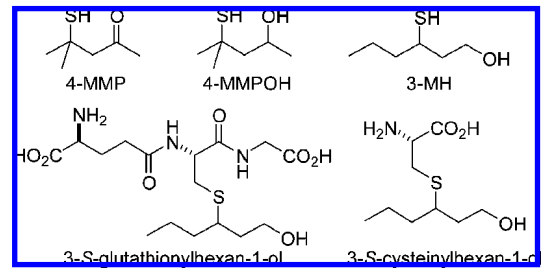

Figure 1. Structures of volatile thiols 4-MMP, 4-MMPOH, and 3-MH found in wine and precursors glut-3MH and cys-3MH found in grape juice.

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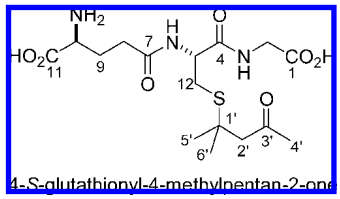

Figure 2. Structure and atom numbering for putative volatile thiol precursor glut-4-MMP.

enous substrates (*9*, *10*). The enzymes responsible for glutathione conjugation have been identified as glutathione *S*-transferases (*10*, *11*). These enzymes catalyze the reaction of electrophiles, such as alkylating agents, with the −SH group of glutathione, thereby neutralizing the reactive sites and rendering the products more water-soluble. Glutathione conjugates are usually unstable and are thought to be metabolized further by cleavage of the glutamate and glycine residues (*9*). However, the biosynthetic pathway for production of *S*-cysteine conjugates of 3-MH, 4-MMP, and 4-MMPOH in grapes has yet to be clarified.

Considering the important contribution of volatile thiols to wine aroma, knowledge about their precursors is vital for gaining an understanding of the pathways to thiol formation during fermentation. While the identification of the glutathione conjugate of 3-MH was described in 2002 as a grape juice component (*7*), no evidence has been reported for the presence of the glutathione conjugates of 4-MMP (glut-4-MMP) or 4-MMPOH in grape juice. The apparent absence of these glutathione conjugates was of interest to us, because these compounds are likely to be related to the biosynthesis of their 4-*S*-cysteinyl congeners and thus to the corresponding free odorants. In the case of 4-MMP in particular, the free thiol has an extremely low reported sensory threshold of 3 ng/L in wine (*1*) and can be found at levels that are important to the sensory attributes and varietal characteristics of a wine.

In this paper, we report the identification of glut-4-MMP (**Figure 2**) as a component of a Sauvignon Blanc juice for the first time based on high-performance liquid chromatography−tandem mass spectrometry (HPLC−MS/MS) techniques and a comparison of data obtained for the juice extract and a reference sample of glut-4-MMP prepared synthetically.

MATERIALS AND METHODS

Chemicals. All chromatographic solvents were HPLC-grade; all chemicals were analytical-reagent-grade unless otherwise stated; and water was obtained from a Milli-Q purification system (Millipore, North Ryde, New South Wales, Australia). Merck solvents were purchased from Rowe Scientific (Lonsdale, South Australia, Australia), and chemicals were obtained from either Sigma-Aldrich (Castle Hill, New South Wales, Australia) or BDH (Kilsyth, Victoria, Australia). All prepared solutions were a percentage (v/v) with the balance made up with Milli-Q water, unless otherwise specified.

Juice Sample. The Sauvignon Blanc juice (2008 vintage; glucose + fructose, 218.5 g/L; pH 3.15; titratable acidity, 7.6 g/L as tartaric acid) came from Tasmania (Australia) and was kept frozen at −20 °C until required.

Synthesis of γ-L-Glutamyl-*S*-(1,1-dimethyl-3-oxobutyl)-L-cysteinylglycine (4-*S*-glutathionyl-4-methylpentan-2-one; glut-4-MMP). To glutathione (2.00 g, 6.5 mmol) in water (13 mL), was added pyridine (1.03 g, 13.0 mmol) and mesityl oxide (0.64 g, 6.5 mmol). The mixture was stirred at room temperature for 45 h before being diluted with water (40 mL) and washed with dichloromethane. The aqueous layer was then concentrated under reduced pressure at 50 °C and 10 mbar using a rotary evaporator. The residue (2.53 g) was recrystallized from a mixture of ethanol (80 mL) and water (6 mL), yielding a white powder (2.13 g, 79%); mp 160−162 °C; $[\alpha]_D$ −9.5 (*c* 0.63, H_2O); ^{1}H NMR (300 MHz, D_2O, δ ppm) 4.56 (1H, dd, J = 7.8 and 5.4 Hz, H_5), 3.95 (2H, s, H_2), 3.82 (1H, t, J = 6.3 Hz, H_{10}), 3.08 (1H, dd, J = 13.2 and 5.4 Hz, H_{12a}), 2.92 (1H, dd, J = 13.2 and 7.8 Hz, H_{12b}), 2.82 (2H, s, $H_{2'}$), 2.60−2.48 (2H, m, H_8), 2.23 (3H, s, $H_{4'}$), 2.16 (2H, app. q, J = ~6.5 Hz, H_9), 1.38 (6H, s, $H_{5',6'}$); ^{13}C NMR (75.5 MHz, D_2O, δ ppm) 213.5 ($C_{3'}$), 175.0, 174.7, 173.6, 172.5 ($C_{1,4,7,11}$), 53.9 (C_{10}), 53.7 ($C_{2'}$), 53.6 (C_5), 44.2 ($C_{1'}$), 41.7 (C_2), 32.0 ($C_{4'}$), 31.4 (C_8), 29.5 (C_{12}), 28.3 ($C_{5',6'}$), 26.2 (C_9); ESI−MS (*m*/*z*) 406.0 $[M + H]^+$, 428.0 $[M + Na]^+$, 444.0 $[M + K]^+$.

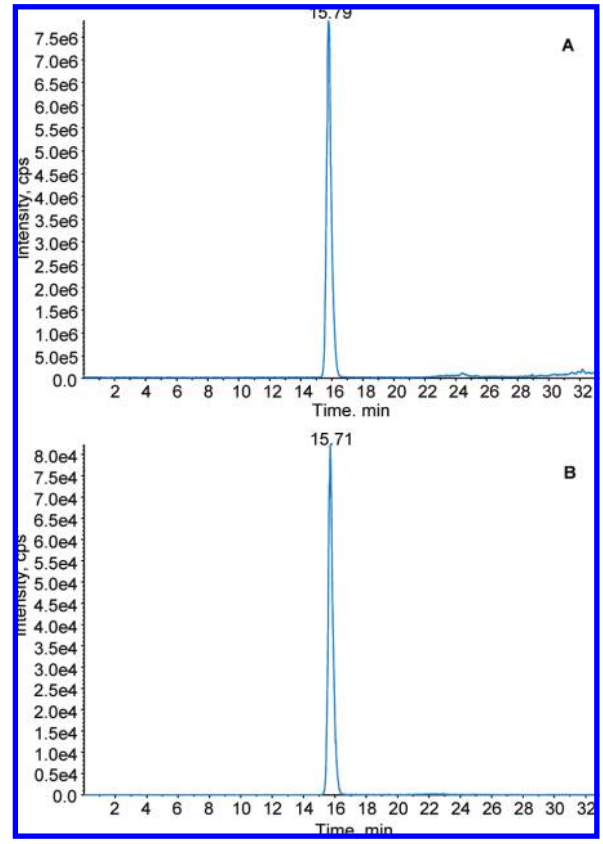

Figure 3. HPLC−MS/MS total ion chromatograms of synthetic glut-4-MMP recorded in (**A**) EPI mode for parent ion *m*/*z* 406 and (**B**) MRM mode for *m*/*z* pairs 406 → 331, 406 → 259, and 406 → 174.

Preparation of Sauvignon Blanc Crude Extract. A total of 6 L of Sauvignon Blanc juice (Tasmania, 2008 vintage) were eluted in batches through a sintered glass funnel (Ø 100 mm, 50 mm bed height) packed with 150 g of C18 sorbent. The stationary phase was previously activated with 100 mL of methanol and then washed with 100 mL of water. After loading the juice (300 mL), the sorbent was rinsed with water (100 mL) and eluted with methanol (100 mL). The stationary phase was re-equilibrated with water (100 mL), and another batch of juice was loaded, with the process being repeated until all of the juice was eluted. The methanol eluates were pooled, and the methanol was removed *in vacuo* on a rotary evaporator at 40 mbar with a 30 °C water bath to yield a final volume of ~200 mL of aqueous juice extract.

This crude aqueous extract (~200 mL) was diluted with water (50 mL), and ~30 mL was applied to a C18 column (250 × 35 mm, 100 mm bed height) previously equilibrated with 100 mL of methanol followed by 100 mL of water. After loading the crude extract, the column was rinsed with water (200 mL) and eluted with methanol (100 mL) using nitrogen gas to provide flow. The colorless water fraction was discarded, while the yellow/orange methanol fraction was retained. The remainder of the crude extract was processed batch-wise in the same manner as above, with the column being re-equilibrated with 100 mL of water after each elution with methanol. The methanol fractions were pooled and concentrated with a rotary evaporator as above, giving a total volume of ~20 mL of a syrupy, light orange-colored juice extract. This extract was diluted 1:9 with water and filtered through a 0.45 μm filter for HPLC−MS analysis.

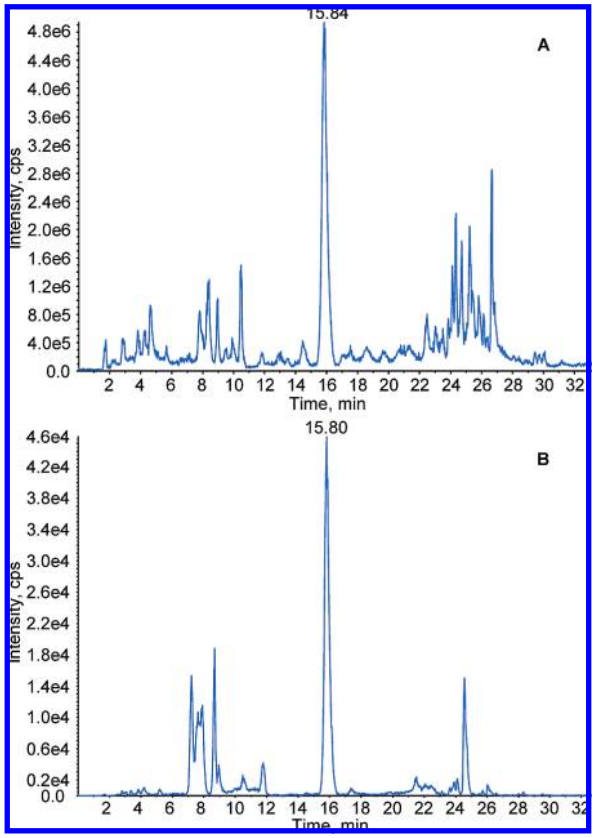

Figure 4. HPLC−MS/MS total ion chromatograms of Sauvignon Blanc juice extract recorded in (**A**) EPI mode for parent ion *m*/*z* 406 and (**B**) MRM mode for *m*/*z* pairs 406 → 331, 406 → 259, and 406 → 174.

HPLC−MS Analysis of Synthetic 4-*S*-Glutathionyl-4-methylpentan-2-one and Sauvignon Blanc Juice Extract. *HPLC−MS Instrumentation.* All HPLC−MS analyses were carried out on an Agilent 1200 instrument (Agilent, Forest Hill, Victoria, Australia) equipped with a binary pump and diode array detector (DAD) connected in series to a 4000 Q Trap hybrid tandem mass spectrometer with a TurboIonSpray source (Applied Biosystems/MDS Sciex, Concord, Ontario, Canada). Data acquisition and processing were performed using Applied Biosystems/MDS Sciex Analyst software (version 1.4.2).

HPLC Conditions. The column was a 250 × 2.1 mm i.d., 5 μm, Alltima C18 (Grace Davison Discovery Sciences, Baulkham Hills, New South Wales, Australia) operated at 25 °C and protected by a 4 × 2 mm i.d. guard column of the same material (Phenomenex, Lane Cove, New South Wales, Australia). The solvents were 0.1% aqueous acetic acid (solvent A) and 0.1% acetic acid in acetonitrile (solvent B), with a flow rate of 0.300 mL/min. The gradient for solvent B was as follows: 0 min, 5%; 10 min, 7%; 15 min, 7%; 32 min, 80%, and 35 min, 90%. The column was equilibrated with 5% B for 10 min prior to an injection. A 10 μL injection volume was used for each sample.

Mass Spectrometer Conditions. All mass spectrometric data were obtained in positive-ion mode. Nitrogen was used for curtain gas (CUR), at 103.4 kPa, nebulizing gas (GS1), at 344.7 kPa, drying gas (GS2), at 344.7 kPa, and collision gas (high). The ion spray voltage, declustering potential, source temperature, and collision energy were set at 5500 V, 60 V, 500 °C, and 30 V, respectively. For enhanced product ion (EPI) experiments, Q1 had unit resolution, the scan rate was set at 1000 amu/s, dynamic fill time was selected for the ion trap, and mass spectra were recorded between *m*/*z* 100 and 500 for a parent ion of *m*/*z* 406.4. For multiple reaction monitoring (MRM), Q1 and Q3 had unit resolution and the transitions chosen were *m*/*z* 406.4 → 331.3, *m*/*z* 406.4 → 259.3, and *m*/*z* 406.4 → 174.2, with a dwell time of 100 ms each. MRM parameters were optimized with infusion MS/MS experiments of a pure synthetic reference compound (2 mg/L) dissolved in water, using an infusion pump operating at 5 μL/min.

Spiking Experiments. The juice extract was diluted 1:9 with water and filtered, and a 200 μL aliquot was used for the spiking experiments. The unspiked sample was analyzed by HPLC−MS using MRM and then re-analyzed after addition of 20 μL of a 2 mg/L solution of the pure synthetic reference compound. This spiking and analysis procedure was repeated with an additional 40 μL, resulting in the addition of 60 μL of reference solution in total. Separately, a 200 μL aliquot of the diluted and filtered juice extract was analyzed by HPLC−MS using MRM after the addition of 200 μL of a 2 mg/L solution of pure synthetic reference compound.

NMR Analysis. Proton (^{1}H) and carbon (^{13}C) nuclear magnetic resonance (NMR) spectra were recorded on a Varian Gemini 300 spectrometer operating at 300 MHz for proton and 75.5 MHz for carbon nuclei. Chemical shifts were recorded as δ values in parts per million (ppm). Spectra were acquired in deuterium oxide (D_2O) at ambient temperature, and resonances were assigned by routine 2D correlation experiments.

RESULTS AND DISCUSSION

Synthesis of 4-*S*-Glutathionyl-4-methylpentan-2-one (glut-4-MMP). Synthesis of the glutathione conjugate of 4-MMP (**Figure 2**) was achieved by a strategy adapted from that reported by Howell et al. for the synthesis of the corresponding cysteine conjugate (*12*). Mesityl oxide was treated with glutathione in water containing 2 equiv of pyridine. The product was isolated and readily purified by recrystallization to give the desired adduct in good yield (79%). Spectroscopic data, reported here for the first time, were in complete accordance with the assigned structure of this compound.

Identification of 4-*S*-Glutathionyl-4-methylpentan-2-one in a Sauvignon Blanc Juice Extract. *Infusion MS/MS and HPLC−MS Analysis of Synthetic Reference Compound.* First, an aqueous solution of synthetic glut-4-MMP (2 mg/L) was infused into the mass spectrometer in positive-ionization mode to obtain and optimize the fragmentation pattern of the analyte by collision-induced dissociation (CID). This resulted in the choice of mass transitions *m*/*z* 406 → 331, 406 → 259, and 406 → 174 and optimization of CID parameters, such as collision energy, for MRM experiments. Next, we recorded the total ion EPI and MRM chromatograms (**Figure 3**) of the glut-4-MMP reference compound. The spectrum taken from the EPI chromatogram displayed the fragmentation of the parent ion *m*/*z* 406. The peaks at *m*/*z* 331 and 259 correspond to the loss of glycine and glutamic acid residues, respectively (see later, **Figure 5**). Repeated analysis of a solution of the reference compound stored at room temperature over the course of a week showed no visible signs of degradation.

Preparation of Sauvignon Blanc Juice Extract. Mass spectra and retention time data for synthesized glut-4-MMP were used to assess the presence of the analyte in the Sauvignon Blanc juice extract, obtained by percolating 6 L of juice through a C18-packed sintered funnel and eluting with methanol. Because of the viscosity of the juice, the process required batch-wise elution to process all of the juice, giving approximately 200 mL of aqueous extract. For further purification, the extract was passed through C18 sorbent, this time using low-pressure column chromatography. Once again, the elution was carried out in a batch-wise manner, to yield approximately 20 mL of concentrated juice extract.

HPLC−MS Analysis of the Juice Extract. Analysis of the Sauvignon Blanc juice extract by HPLC−MS revealed the presence of glut-4-MMP after a comparison to data from the synthetic reference compound. Initially, a total ion chromatogram in scan mode was obtained, and the extracted ion chromatogram for *m*/*z* 406 displayed a peak at the correct retention time of 16 min (data not shown). Further HPLC−MS/MS analyses involved obtaining the total ion EPI and MRM

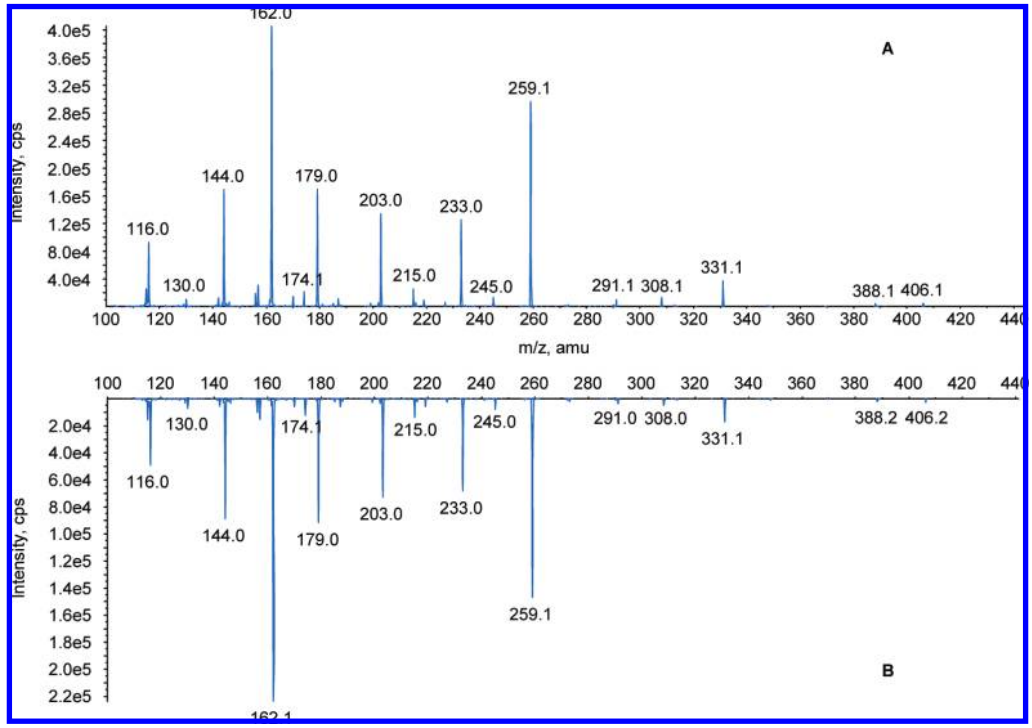

Figure 5. Comparison of mass spectra of the 16 min peak obtained from EPI experiments of parent ion *m*/*z* 406 for (**A**) synthetic reference sample of glut-4-MMP and (**B**) Sauvignon Blanc juice extract.

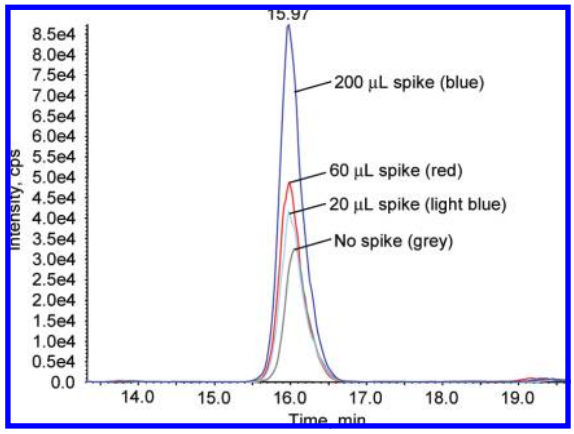

Figure 6. Overlaid and expanded total ion MRM chromatograms for Sauvignon Blanc juice extract spiked with increasing levels of synthetic reference compound.

chromatograms (**Figure 4**), where the retention times and, more importantly, the EPI mass spectrum of parent ion *m*/*z* 406 for glut-4-MMP (**Figure 5**) were in total agreement with those of the synthetic reference compound. No carry-over of glut-4-MMP was evident between HPLC−MS injections at the concentrations analyzed.

HPLC−MS Analysis of Spiked Juice Extract. For further confirmation of the presence of glut-4-MMP in the Sauvignon Blanc juice, co-injection experiments of the juice extract and reference compound were carried out. The juice extract, spiked with increasing amounts of reference compound, was analyzed by HPLC−MS/MS in MRM mode, and these analyses provided additional evidence for the presence of glut-4-MMP in the juice. Each addition of the reference compound symmetrically enhanced the peak at 16 min (**Figure 6**), but no other peaks were similarly affected. On the basis of these results, in conjunction with the other HPLC−MS experiments and a comparison to an authentic sample, the presence of glut-4-MMP in a grape juice extract was clearly demonstrated for the first time. To the best of our knowledge, this is also the first time this compound has been identified as a natural product. From the various experiments, it was estimated that this particular Sauvignon Blanc juice sample had a glut-4-MMP concentration of approximately 5 μg/L.

This result furthers our understanding of the presence of putative volatile thiol precursors and complements previous studies that identified other conjugated precursors in grape juices. This information will aid future studies aimed at determining the inter-relationships between the thiol precursors found in grapes and juice and the resulting aroma-active compounds in wine. For instance, enzymatic release of 4-MMP from its glutathione conjugate may provide a measure of the aroma potential of a juice similar to the work of Peyrot des Gachons et al. on cysteine conjugates (*13*). Moreover, because the *S*-cysteinyl conjugates are always found along the metabolic pathway of the degradation of the relevant *S*-glutathionyl conjugates, these glutathione derivatives might be the real precursors to a range of compounds important to wine sensory properties. Indeed, Subileau et al. alluded to the role of glutathione conjugates in their recent work on the biogenesis of 3-MH in Sauvignon Blanc wines (*14*), which tends to support this view.

ABBREVIATIONS USED

glut-4-MMP, 4-*S*-glutathionyl-4-methylpentan-2-one; 4-MMP, 4-mercapto-4-methylpentan-2-one; 4-MMPOH, 4-mercapto-4-methylpentan-2-ol; 3-MH, 3-mercaptohexan-1-ol; glut-3-MH, 3-*S*-glutathionylhexan-1-ol; cys-3-MH, 3-*S*-cysteinylhexan-1-ol; EPI, enhanced product ion; MRM, multiple reaction monitoring; CID, collision-induced dissociation.

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